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REPORT TITLE: Characterization of Monoclonal Antibodies From Hybridomas BE2 and HA4

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ABSTRACT

This report describes the use of an Enzyme-Linked Immunosorbent Assay (ELISA) to screen several samples of monoclonal antibody containing (MCA) spent culture medium from hybridomas that were produced by Litron Laboratories LTD. These MCA containing samples were derived from mice that were inoculated with succinylated-hydroxyethylnicotine conjugated to the carrier protein human serum albumin. The antibody containing supernatants were sent to Philip Morris R&D where they were screened using an ELISA procedure that was developed by Project 6902. The ELISA allows us to measure the amount of the antibody present in the culture media as well as to run the culture media in a nicotine and nicotine analog inhibition ELISA. There were two samples of antibody containing culture media that resulted in good titers, high nicotine affinity, and low cross reactivity with nicotine analog compounds. These samples are spent culture media from hybridomas designated BE2 and HA4. This report describes the data related to these two samples, BE2 and HA4, demonstrating that they indeed can be used to detect free nicotine in solution.

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I. INTRODUCTION

One of Project 6902's goals has been to introduce antibody based ELISA technologies into PM laboratories and implement or develop assays for compounds of interest. The initial effort in this area has centered around the development of an antibody based ELISA to detect and quantitate nicotine concentrations in tobacco extracts. This report will detail the efforts which have been made to obtain nicotine specific antibodies and to develop a nicotine ELISA based on those antibodies.

The work described in this report is completely dependent upon the generation of antibodies and the subsequent utilization of their unique properties. Antibodies are generated during the immune response which occurs when animals are subjected to challenges by substances foreign to the body.

When a foreign substance enters the body, it initiates a response. This response includes the production of very specialized protein molecules, referred to as antibodies, which bind to that substance and facilitate its neutralization and elimination. These proteins are produced by lymphocytes which individually respond to the foreign substance, or antigen, by producing a unique antibody to that material. The antibodies produced by each individual lymphocyte will only recognize and bind to one specific site on the antigen. These sites are referred to as the antibody's antigenic determinant. Due to the large number of lymphocytes which respond to each antigenic challenge the normal response of an animal is to produce a very heterogeneous

antibody population directed against that antigen. These antibodies are secreted by the lymphocytes into the blood from which they can be collected, and their unique and specific binding characteristics exploited. Since these antibodies isolated from blood are the product of many lymphocytes, they are referred to as polyclonal antibodies (PCA).

While PCAs are specific for various classes of antigenic compounds, they do not usually display a high specificity for analogs of the same class as the antigen. Recently however, several new techniques have been developed which can allow the preparation and isolation of the antibodies against a single antigenic determinant. To prepare these antibodies, lymphocytes are removed from immunized animals and individually fused, or hybridized, with a type of cell which can be maintained in culture in the laboratory. Using standard cell culture techniques., these "hybrid" cells, referred to as hybridomas, are sub-cloned into individual cell lines, each of which retains the ability to produce the same antibody as the original lymphocyte. These individual cell cultures are then screened biochemically to determine which secrete antibodies with the desired specificity. Since the antibodies obtained using this method are derived from a single lymphocyte cell line they are referred to as monoclonal antibodies (MCA).

Traditional bioanalytical techniques have utilized the specific, high affinity binding characteristics of antibodies to develop biochemical assays. These biochemical assays have been used to augment or replace many classical chemical analytical procedures. For example, MCA based enzyme-linked immunosorbent assays (ELISA) currently exist to analyze for the presence

and concentration of several plant hormones (1), nicotine (2), and methoprene (3) associated with tobacco.

While nicotine does have several antigenic determinants, it is too small a molecule to be recognized by the immune system and stimulate the synthesis of antibodies. As such, the initial portion of this research necessitated the synthesis of nicotine-protein conjujugates. One such conjugate, succinylated hydroxyethylnicotine conjugated to albumin (SHN-HSA), was required for immunization and a second, succinylated hydroxyethylnicotine conjugated to thyroglobulin (SHN-TG), for screening for the production of nicotine specific antibodies. The immunization conjugate was sent to a commercial laboratory for the actual production of MCAs. Once prepared, the MCAs were sent to PM laboratories where they were screened for desirable binding characteristics using ELISAs.

To conduct an ELISA assay, the compound of interest is usually directly adsorbed onto a specially treated microtiter plate. However, in some instances, for example when the compound is of very low molecular weight such as nicotine, it is chemically attached to a protein which can subsequently bind to the plate. In our assay for example, SHN-TG is used for this purpose. The SHN-TG conjugate was used because any antibodies which had been generated against the albumin (HSA) portion of the conjugate would bind to HSA attached to the plate and appear as a false positive nicotine selective antibody. The primary antibodies, those which have been developed against the nicotine, are then added to the plate and allowed to interact with and bind to the appropriate site on SHN-TG. Following an extensive washing to remove any primary

antibodies which are not bound to SHN-TG, a specially modified secondary antibody is introduced. This secondary antibody, usually obtained from commercial sources, has been developed such that it will only recognize and bind to any primary antibodies which remain bound to SHN-TG attached to the plate. In addition, these antibodies have been biochemically modified so that they can be detected following their binding to the primary antibody using a general detection method. To facilitate this detection, the secondary antibody had been biochemically modified to contain a hydrolytic enzyme. After the reaction plate has been washed to remove unbound secondary antibody, the enzyme's substrate is added and allowed to react. Within this particular ELISA, the enzyme hydrolyzes the colorless substrate into a colored product whose concentration can be determined spectrophotometrically at 405 nm. Presence of absorbance at 405 nm (A405) indicates the presence of the secondary antibody which in turn indicates the interaction of primary antibody with SHN-TG attached to the plate.

This procedure is commonly used to screen for MCAs which recognize and bind to the antigen of interest. However, it can also be modified to quantitate the concentrations of nicotine present in test samples. In this type of assay, small amounts of the solution of interest are incubated with the primary antibody prior to its addition to the plate. If the solution contains nicotine, it will bind to the primary antibody and prevent its interaction and binding to the nicotine conjugate (SHN-TG) on the plate. This results in less primary antibody becoming bound to the plate and ultimately less secondary antibody binding and enzyme activity present. Following incubation with the enzyme substrate, a lower A405 will be detected. It can be

inferred that a negative correlation will exist between the concentration of nicotine in the test sample and the A405. Following the construction of a standard curve determined by adding known amounts of nicotine and determining the resultant A405, the concentration of nicotine in unknown solutions can be determined.

The remainder of this report will discuss the synthesis and characterization of SHN-TG and SHN-HSA, the production of MCAs by an external contract laboratory, the subsequent selection and characterization of resultant nicotine specific MCAs, and the development of a nicotine ELISA.

II. MATERIALS AND METHODS

A. Chemicals

1. Succinylated Hydroxyethylnicotine (SHN) obtained from Dr. J. Seeman and Mr. H. Secor of the Chemical Research Division (4).

2. Human Serum Albumin (HSA), No. A-3782, Sigma Chemical Co., St. Louis, MO. 63178. Store at -20°C.

3. Bovine Thyroglobulin (TG), No. T-1001, Sigma Chemical Co., St. Louis, MO. 63178. Store at -20°C.

4. Sodium Carbonate, No. S-263, Fisher Scientific, Fair Lawn, NJ. 07410. Store at room temperature.

5. Sodium Bicarbonate, No. S-233, Fisher Scientific, Fair Lawn, NJ. 07410. Store at room temperature.

6. Knox Gelatin, Food Grade. Knox Gelatin Inc., Englewood Cliffs, NJ. 07632. Store at room temperature.

7. Dulbecco's Phosphate-Buffered Saline (D-PBS), No. 310-4190AJ, Gibco Laboratories, Life Technologies, Inc., Grand Island, NY. 14072. Store at room temperature.

8. Anti-mouse IgM (μ-chain specific) Alkaline Phosphatase Conjugate, No. A-7784, Sigma Chemical Co., St. Louis, MO. 63178. Store at 4°C.

9. Anti-mouse IgG (whole molecule) Alkaline Phosphatase Conjugate, No. A-5781, Sigma Chemical Co., St. Louis, MO. 63178. Store at 4°C.

10. Sodium Chloride, No. 3624-1, J.T. Baker Chemical Co., Phillipsburg, NJ. 08865. Store at room temperature.

11. Potassium Chloride, No. P-217, Fisher Scientific, Fair Lawn, NJ. 07410. Store at room temperature.

12. Sodium Phosphate, Dibasic, No. 3828-1, J.T. Baker Chemical Co, Phillipsburg, NJ. 08865. Store at room temperature.

13. Potassium Phosphate, No. P-288 Fisher Scientific, Fair Lawn, NJ. 07410. Store at room temperature.

14. Tween-20, No. 170-6531, Bio-Rad Laboratories, Richmond, CA. 94804. Store at room temperature.

15. 2-Amino-2-methyl-1-propanol buffer (AMP buffer), No. 221, Sigma Chemical Co., St. Louis, MO. 63178. Store at 4°C.

16. Magnesium Chloride, No. M-33, Fisher Scientific, Fair Lawn, NJ. 07410. Store at room temperature.

17. p-Nitrophenyl phosphate (pNPP), No. N9389, Sigma Chemical Co., St. Louis, MO. 63178. Store at -20°C.

18. 1-Ethyl-3-[3-Dimethyl-Aminopropyl]Carbodiimide (EDAC), No. E 6383, Sigma Chemical Co., St. Louis, MO. 63178. Store at -20°C.

19. l-Nicotine free base #8325-59a obtained from Dr. Jeff Seeman and Mr. Henry Secor (5).

20. Nicotine Analogs obtained from Dr. Jeff Seeman and Mr. Henry Secor (6).

a. R,S Nornicotine, file #185, Mol. Wt. 148, Notebook #8079-77b.

b. Anabasine, file #229, Mol. Wt. 162, Notebook #7566-168a.

c. d,l Anatabine, file #581, Mol. Wt. 160, Notebook #8079-67b.

d. Nicotinic Acid, No. N-785-0, Aldrich Chemical Company, Inc., Milwaukee, WI. 53233.

e. Cotinine, file #630, Mol. Wt. 176, Notebook # 8079-89a.

f. Trans-Nicotine-N'-Oxide, file #677, Mol. Wt. 178, Notebook # 8325-37b.

g. 2,3'-Dipyridyl, No. 19-888-9, Aldrich Chemical Company, Inc., Milwaukee, WI. 53233.

h. Nicotyrine, file #21, Mol. Wt. 158, Notebook #7957-2c.

i. Myosmine, file #406, Mol. Wt. 146, Notebook #8079-11a.

j. N'-Methylanabasine, file #55, Mol. Wt. 176, Notebook #7228-14a.

21. Mili Q Reagent water (HQH2O), Millipore Corporation, Bedford, MA. 01730.

B. Equipment

1. Rainin Pipettor (1000 µl), #P1000, Rainin Instrument Co., Woburn, MA. 01801.

2. Rainin Pipettor (100 µl), #P100, Rainin Instrument Co., Woburn, MA. 01801.

3. Belly Dancer Shaker, Stovall Life Sciences Inc., Greensboro, N.C.

4. Hitachi 150-20 Spectrophotometer, Hitachi Instruments, Inc., Danbury, CT. 06810.

5. Fisher Isotemp Incubator, Fisher Instruments.

6. Bio-Tek Autoreader, Model EL310, Bio-Tek Instruments, Inc. Winooski, VT. 05404.

7. Immulon 4 Flat Bottom Plates, No. 011-010-3850, Dynatech Laboratories, Inc., Chantilly, VA. 22021.

8. Centricon 30 Microconcentrator, #4209, Amicon Division, W. R. Grace Co., Danvers, MA. 01923.

9. 2.0 ml Microfuge Tubes, No. 500 72.689, Sarstedt Laboratory, West Germany.

10. 50 ml Centrifuge Tubes, No. 000-2091-STR, Elkay Products, Inc., Shrewsbury, MA. 01545.

11. 15 ml Graduated Conical Tube, No. 2097, Becton Dickinson and Co., Lincoln Park, NJ. 07035.

12. Corning Hot Plate-Stirrer, No. PC-351, Baxter Healthcare Corp., Columbia, MD. 21045.

C. Solutions

1. Carbonate/Bicarbonate Coating Buffer Combine 1.59 gms of sodium carbonate, and 2.93 gms of sodium bicarbonate. Adjust the pH of this solution to 9.6 with HCl and bring up to 1 liter with distilled deionized water. Store at 4°C.

2. Phosphate Buffered Saline-Tween (PBS-Tween) Combine 8.0 gms of NaCl, 0.2 gms of potassium phosphate, 2.9 gms of sodium phosphate dibasic, 0.2 gms of potassium chloride, and 0.5 ml of Tween 20. Adjust the pH of this solution to 7.4 with HCl and bring up to 1 liter with distilled deionized water. Store, at 4°C.

3. 1% Knox Gelatin Add 5.0 gms of Knox gelatin to 500 ml of D-PBS. Dissolve by heating approximately 30 minutes on a hot plate at a setting

of 5 with stirring. Let cool to room temperature. Prepare this solution fresh on the day of the experiment.

4. Anti-mouse IgM (Secondary Antibody IgM) Combine 10 ml of 1% Knox gelatin and 10 µl of anti-mouse IgM in a 15 ml graduated conical tube. Prepare this solution fresh immediately prior to use.

5. Anti-mouse IgG (Secondary Antibody IgG) Combine 10 ml of 1% Knox gelatin and 10 µl of anti-mouse IgG in a 15 ml graduated conical tube. Prepare this solution fresh immediately prior to use.

6. 1M Magnesium Chloride Dissolve 101.66 gms of magnesium chloride in sufficient distilled deionized water to give a final volume of 500 ml. Store at 4°C.

7. AMP Buffer Combine 670 µl of 2-amino-2-methyl-1-propanol buffer and 100 µl of 1 M magnesium chloride. Add sufficient distilled deionized water to give a final volume of 100 ml. Store at 4°C.

8. Substrate Solution (pNPP) Combine 10 ml of AMP buffer and two 5 mg pNPP tablets (5 mg tablet/5 ml AMP buffer). Prepare this solution fresh immediately prior to use.

9. Secondary Antigen (SHN-TG) Combine 50 µl of a 2 mg/ml stock solution of SHN-TG or TG to 10 ml of carbonate/bicarbonate coating buffer. Prepare this solution fresh on the day that the microtiter plate is coated.

10. Nicotine in Solution Prepare a 1E-2 M stock solution by adding 16.2 µl of nicotine free base to 10 ml of D-PBS. Prepare concentrations 1E-4-1E-8 M in D-PBS using this stock. Store at 4°C.

11. R,S Nornicotine Add 100 µl of R,S nornicotine free base to 358 µl of D-PBS to prepare a 1mM stock solution. Prepare concentrations 1E-4 - 1E-8 M in D-PBS using this stock. Store at 4°C.

12. Anabasine Add 100 µl of anabasine free base to 3.6 µl of D-PBS to prepare a 1 mM stock solution. Prepare concentrations 1E-4 - 1E-8 M in D-PBS using this stock. Store at 4°C.

13. d,l Anatabine Add 100 µl of d,l anatabine free base to 3.75 µl of D-PBS to prepare a 1 mM stock solution. Prepare concentrations 1E-4 to 1E-8 M in D-PBS using this stock. Store at 4°C.

14. Nicotinic Acid Add 100 µl of nicotinic acid free base to 585 µl of D-PBS to prepare a 1 mM stock solution. Prepare concentrations 1E-4 - 1E-8 M in D-PBS using this stock. Store at 4°C.

15. Cotinine Add 100 µl of cotinine free base to 210 µl of D-PBS to prepare a 1 mM stock solution. Prepare concentrations 1E-4 to 1E-8 M in D-PBS using this stock. Store at 4°C.

16. Trans-Nicotine-N-Oxide Add 100 µl of trans-nicotine-N-oxide free base to 161 µl of D-PBS to prepare a 1 mM stock solution. Prepare concentrations 1E-4 - 1E-8 M in D-PBS using this stock. Store at 4°C.

17. 2,3' Dipyridyl Add 100 µl of 2,3' Dipyridyl free base to 284 µl of D-PBS to prepare a 1 mM stock solution. Prepare concentrations 1E-4 - 1E-8 M in D-PBS using this stock. Store at 4°C.

18. Nicotyrine Add 100 µl of nicotyrine free base to 384 µl of D-PBS to prepare a 1 mM stock solution. Prepare concentrations 1E-4 - 1E-8 M in D-PBS using this stock. Store at 4°C.

19. Myosmine Add 100 µl of myosmine free base to 943 µl of D-PBS to prepare a 1 mM stock solution. Prepare concentrations 1E-4 - 1E-8 M in D-PBS using this stock. Store at 4°C.

20. N'-Methylanabasine Add 100 µl of N'-methylanabasine free base to 192 µl of D-PBS to prepare a 1 mM stock solution. Prepare concentrations 1E-4 - 1E-8 M in D-PBS using this stock. Store at 4°C.

21. 1-Ethyl-3-[3-Dimethyl-Aminopropyl]carbodiimide (EDAC) Dissolve 50 mg of EDAC in 2.0 ml of distilled deionized water, adjust pH to 6.8.

22. Bovine Thryoblobulin (TG) Dissolve 100 mg of TG in 1.0 ml of distilled deionized water, adjust pH to 6.8.

23. Succinylated Hydroxyethylnicotine (SHN) Dissolve 25 mg of SHN in 100 µl of distilled deionized water and adjust pH to 6.7 with NaOH and then bring up to 1.0 ml with distilled deionized water.

24. Succinylated Hydroxyethylnicotine Conjugated to Human Serum Albumin (SHN-HSA) (Primary Antigen) A solution of SHN-HSA (primary antigen) was prepared and an aliquot sent to Litron Laboratories LTD in Rochester, NY (7). Project 6902 contracted Litron Labs to conduct the production of the monoclonal antibodies. A summary of the procedure that was used to produce the hybridomas from which our antibodies were obtained is found in **Appendix 1.**

D. Methods

1. Reaction Mixture Preparation for the Synthesis of SHN-TG (Secondary Antigen)

(a) Make up the following reactions by combining:

1/4 mg/mg SHN/TG	TG
500 µl TG (100 mg/ml)	500 µl TG (100 mg/ml)
500 µl SHN (25 mg/ml)(pH 6.8)	500 µl HQH2O (pH 6.8)
1000 µl	1000 µl

(b) Add dropwise with mixing 1000 µl EDAC (25 mg/ml) pH 6.8.

(c) Incubate reaction overnight with shaking at room temperature.

(d) Place sample in a Centricon 30 and wash 3 times with 1 ml aliquots of distilled deionized water.

(e) Bring to void volume of 300 µl.

(f) Using 10 µl of each reaction mixture dilute to 100 µl in distilled deionized water and run a BIORAD Protein Assay using 5 mg/5 ml TG solution as standard. Dilute the TG standard to 1000, 750, 500, 250 and 0 µg/ml using distilled deionized water. Measure the amount of protein in the samples.

(g) Bring volume of the sample up to 2.5 ml with D-PBS.

(h) Store reaction solution in a labeled 15 ml graduated conical tube at 4°C.

3. Antibody Titer

Several microtiter plates may be prepared and stored up to 7 days at 4°C.

(a) Preparation of the microtiter plate

(1) Dilute an aliquot of both the SHN-TG (secondary antigen) and TG to a concentration of 1.0 µg/ 100 µl in carbonate/bicarbonate coating buffer.

(2) Pipet 100 µl of the SHN-TG into wells A1-G4 of an Immulon 4 plate, 100 µl of the TG into wells A5-G8, and then coating buffer into wells A9-G12 of the plate.

(3) Cover the plate with Parafilm® and store at least 24 hours at 4°C.

(b) Sample preparation

(1) Prepare dilutions (1/10 - 1/10,000) of each of the spent culture media samples from the hybridomas in D-PBS.

(c) ELISA procedure

(1) Remove the microtiter plate from storage at 4°C.

(2) Empty the plate into the sink and bump it, upside down on paper towels to remove all of the SHN-TG solution.

(3) Completely fill every well of the plate with 1% Knox gelatin solution, cover with Parafilm® and incubate for 1 hour at 37°C.

(4) Remove plate, empty, and wash 3 times with 1% Knox gelatin by filling all the wells completely.

(5) Add the samples as prepared in section B.

(6) Add D-PBS to remaining wells.

(7) Incubate the plate at 37°C for 2 hours.

(8) Remove and wash 3 times by filling all the wells completely with 1% Knox gelatin.

(9) Add 100 µl of anti-mouse IgG or anti-mouse IgM to wells containing sample.

(10) Incubate the plate at 37°C for 2 hours.

(11) Remove plate, empty, and wash 6 times with PBS-Tween by completely filling all the wells.

(12) Add 100 µl of pNPP to all the wells of the plate, cover with Parafilm®, and incubate at 37°C for 30 minutes.

(13) Determine the absorbance of the individual wells of the microtiter plate by reading the plate at 405 nm.

The absorbance of the individual wells of the microtiter plate was determined on the Bio-Tek Autoreader at 405 nm. Each of the absorbances are plotted as a percentage of the maximum response obtained in the assay. The percent maximal response is plotted on the y axis and the log of the dilution on the x axis. The titer which is defined as the dilution which results in one-half the maximal response is obtained from this information.

4. Antibody Class

Antibodies produced using this type of procedure can be of several different classes. In order to determine the class of antibody that is present in the spent culture media from the hybridomas, an anti-mouse IgM and an anti-mouse IgG secondary antibody were

used in the ELISA. These secondary antibodies will identify and attach to specific classes of antibodies within the sample, thereby, determining the class of antibody present in the samples. The samples were classified as being of the IgM or IgG class according to the predominating response using each respective secondary antibody.

5. Nicotine Inhibition

(a) Plate preparation

(1) Dilute an aliquot of SHN-TG to a concentration of 1.0 µg/ 100 µl in coating buffer.

(2) Add 100 µl to all of the wells of a microtiter plate, cover with Parafilm®, and store at least 24 hours at 4°C.

(b) Sample preparation

(1) Dilute each of the spent culture samples (if necessary) in D-PBS such that when diluted in half will give a final absorbance of 2.0 OD at 405 nm in the ELISA assay.

(c) Reaction mixture

(1) Combine 70 µl of each of the spent culture samples with 70 µl of each of the standard nicotine solutions (1E-4 - 1E-8 M) in microfuge tubes.

(2) Vortex, and incubate at room temperature for 30 minutes.

(d) ELISA procedure

(1) Complete the assay as specified in section 3.C.1-13.

The data obtained from reading the absorbance of the wells of this plate at 405 nm was used to plot the absorbance on the y axis and the log of the concentration (μM) of the nicotine on the x axis. The Inhibition Constant was determined using this information. The Inhibition Constant at 50% (IC50) is defined as that concentration of nicotine which causes a one half maximum response.

6. Nicotine Analog Cross Reactivity

(a) Plate preparation

(1) Dilute the SHN-TG to a concentration of 1.0 μg/ 100 μl.

(2) Add 100 μl to all the wells of a microtiter plate, cover with Parafilm®, and store at least 24 hours at 4°C.

(b) Sample preparation

(1) Dilute each of the spent culture samples (if necessary) in D-PBS such that when further diluted in half will give a final absorbance of 2.0 OD at 4.5 nm in the ELISA assay.

(c) Reaction mixture

(1) Combine 70 μl of each of the spent culture samples with 70 μl of each of the nicotine analogs at the 1E-4 to 1E-8 M concentrations.

(2) Vortex and incubate at room temperature for 30 minutes.

(d) ELISA procedure

(1) Complete the assay as specified in Section 3.C.1-13.

The absorbance of the wells of the plate was determined at 405 nm and the data graphed using the standard RS1 procedure. The concentration of the nicotine analogs verses the maximal response plotted as a percent of the maximum absorbance was graphed. The IC50 is calculated for any of the analogs that reacted with the samples. The Inhibition Constant at 50% (IC50) is defined as that concentration of nicotine analog which causes a one half maximum response.

III. RESULTS

A. Titer of the Spent Culture Media Samples Containing Monoclonal Antibodies

Approximately 40 samples were sent to Philip Morris from Litron Labs LTD, Rochester, NY, where the production of the monoclonals was conducted. Two of these samples exhibited a high titer, high affinity towards nicotine, and low cross reactivity with nicotine analogs. These two samples were spent culture media from fusion clones BE2 and HA4. This report will focus on the results from work that has been conducted with these samples which we will refer to as BE2 and HA4.

An ELISA assay was conducted to titer the spent culture media from fusion clone BE2. The sample was serially diluted from 1/10 - 1/5000 in D-PBS and an aliquot of each dilution run in the assay using SHN-TG, TG, and coating buffer. The titer was determined to be

4.6E-3 (see Fig.1). The antibody type was determined by ELISA using both anti-mouse IgG and anti-mouse IgM in the assay. BE2 was found to be of the IgM antibody class (8).

A sample of spent culture media from fusion clone HA4 was also titered. This sample was diluted 1/10 - 1/1000 and run in an ELISA. The titer for HA4 was determined to be 3.16E-3 (see Fig.2). Following testing with each of the secondary antibodies the sample was classified as being of the IgG antibody class (9).

B. Nicotine Inhibition of Spent Culture Media Samples From Fusion Clone BE2 and HA4

An experiment was conducted to examine the nicotine affinity of BE2. In this experiment spent culture media from fusion clone BE2 was diluted 1/160 and combined with dilutions of nicotine in solution. The data from this experiment can be seen in Fig.3. The Inhibition Constant (IC50) of nicotine for BE2 was determined to be 5.75E-5 M (8).

When HA4, at a dilution of 1/400, was combined with the various concentrations of nicotine in solution, an IC50 of 3.98E-6 M (Fig.4) (10) was observed which is approximately a log higher affinity than BE2.

C. Nicotine Analog Cross Reactivity of Spent Culture Media Samples From Fusion Clone BE2 and HA4

In these experiments the cross reactivity of BE2 and HA4 with 10 nicotine analogs was examined. The analogs that were tested with the samples were: R,S nornicotine,

anabasine, d,l anatabine, nicotinic acid, cotinine, trans-nicotine-N-oxide, 2,3' dipyridyl, nicotyrine, myosmine, and N'-methylanabasine. Each of the analogs was diluted to concentrations of 1E-3 to 1E-8 M with D-PBS and an aliquot of each combined with an equal volume of BE2. With the exception of N'-methylanabasine, none of the compounds exhibited any significant cross-reactivity at concentrations below 10^{-3}M. When examined in an extended test, N'-methylanabasine inhibited BE2 monoclonal antibodies in a dose-dependent manner with an IC50 of 3.16E-4 M (Fig. 5) (8).

In the same type of experiment only N'-methylanabasine was found to cross-react with HA4 with an IC50 of 2.50E-8 M (Fig. 6) (11).

IV. DISCUSSION

The ELISAs that were conducted on each of the spent culture media from all fusion clones were reviewed and those that yielded high enough titers with little or no interaction with the thyroglobulin were further tested. Of the approximately 40 clones that we received only the spent culture media from fusion clones BE2 and HA4 were deemed acceptable due to their high titer, low cross-reactivity with the nicotine analogs, and their high affinity towards nicotine. Since HA4 has a log higher affinity for nicotine than does BE2, it has been used to run further experiments to validate our standard ELISA protocol.

VI. ACKNOWLEDGMENTS

The author would like to thank the following people for their assistance that she received during the preparation of this report and the research upon which it is based: Dr. Bruce Davies, Mr. Robert Dunn, Ms. Beverly Vaughan, Dr. Jeff Seeman, and Mr. Henry Secor.

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APPENDIX 1

Litron
Laboratories
Limited

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Summary - Operations Involved in the Philip Morris Hybridoma Project.(1990)

Hapten was conjugated to carrier protein at the Philip Morris Research Center. Human serum albumin (HSA) and thyroglobulin were used as carriers. Initially, hapten-HSA was emulsified with complete Freund's adjuvant and was injected IP into female Balb/c mice. The mice received additional doses of the hapten-HSA conjugate at 3 week intervals. For cell fusion, the spleen was asceptically removed from an immunized mouse and the cells were teased into Dulbecco's Modified Eagle's Medium (DMEM). These cells were fused with myeloma cells (NS1) which were grown in culture. Hybridoma formation was initiated by pelleting the spleen and myeloma cells and resuspending them in a polyethylene glycol solution prepared in DMEM by the method of G. Kohler and C. Milstein (Nature 256, 495 (1975), Eur. J. Immunol. 6, 511 (1976)). The cells were transferred to a Hypoxanthine, Aminopterin, Thymidine (HAT) selection medium. In the following days, the spleen cells die naturally, and the unfused myeloma cells are destroyed by the HAT medium. Only hybridomas which contain the genetic material of both the spleen and myeloma cells will grow. Under our accelerated growth conditions, the wells can be scored for colonies by day 5, and they can be assayed by ELISA assay by day 9 post fusion. In order to determine which cells are producing antibodies against the hapten, the second carrier protein (i.e. hapten-thyroglobulin (THG)) was used in the Enzyme-Linked ImmunoSorbant Assay (ELISA). Specifically, hapten-THG was adsorbed to 96 well vinyl assay plates. Supernatants (50-100 µl) from the wells that contained clones were then transferred to the corresponding wells in the assay plates. Following incubation, the wells were washed and a solution of anti-IgG and anti-IgM Goat AntiMouse Antibody (GAMA) conjugated with alkaline phosphatase was added to each well. Following incubation, the wells were washed to remove any unbound GAMA, and a solution of substrate (p-nitrophenyl phosphate) was added. Those wells that contained antihapten antibodies turned yellow due to the antigen initiated accumulation of alkaline phosphatase in the wells. The positive wells were expanded to 2ml and were reassayed by day 14. Positive supernatants were then sent to Philip Morris for further evaluation against other endpoints in order to determine which clones are producing antibodies that would be useful for their specific projects. In some cases, it was necessary to provide larger volumes of supernatant from select clones for a more rigorous evaluation. In other cases, ascities fluid was chosen as the source of antibody. With ascities fluid the yield of antibody can reach 5 -10 mg/ml and can provide a large amount of a good antibody for further study.

It should be noted that Litron Laboratories has also developed proprietary hybridoma technology that has been used in some parts of the project. This methodology permits us to increase the number of activated lymphocytes, and increase the yield of hybridomas following cell fusion. This technology reduces the amount of antigen needed during the immunization phase of the project. Other aspects of our technology reduce the loss of hybridomas during the subcloning stage, where good antibody producers often die. As a consequence, we are able to provide good yields of antigen specific antibody. Select hybridomas from this project have been frozen in liquid nitrogen for permanent storage and can be revived as they are needed.

Submitted by,

Andrew M. Tometsko

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Director of Research

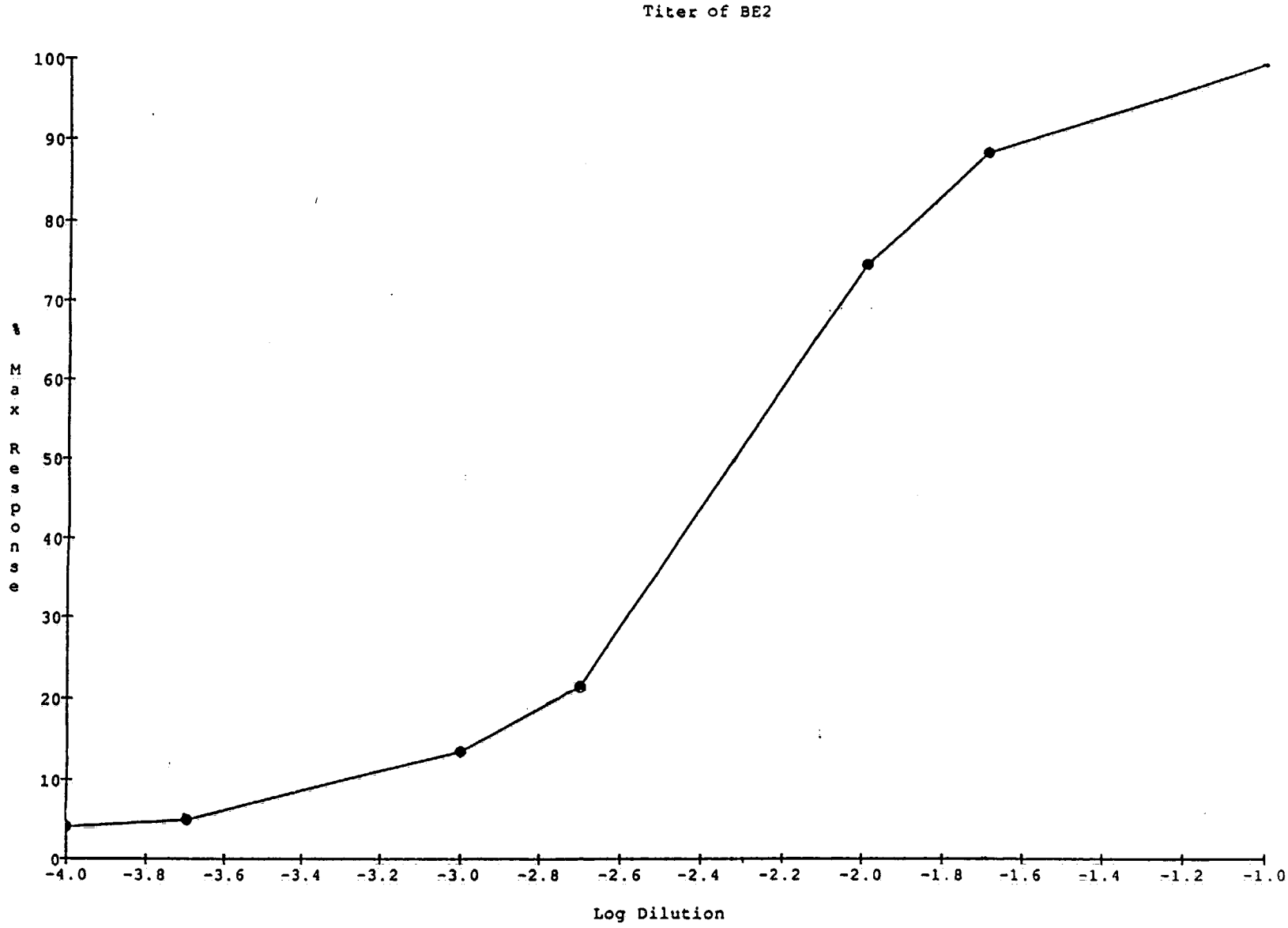

FIGURE 1

Graphical representation of the absorbance of each BE2 dilution plotted as its percent of the maximum absorbance (determined in the absense of nicotine) versus the log of the sample dilution.

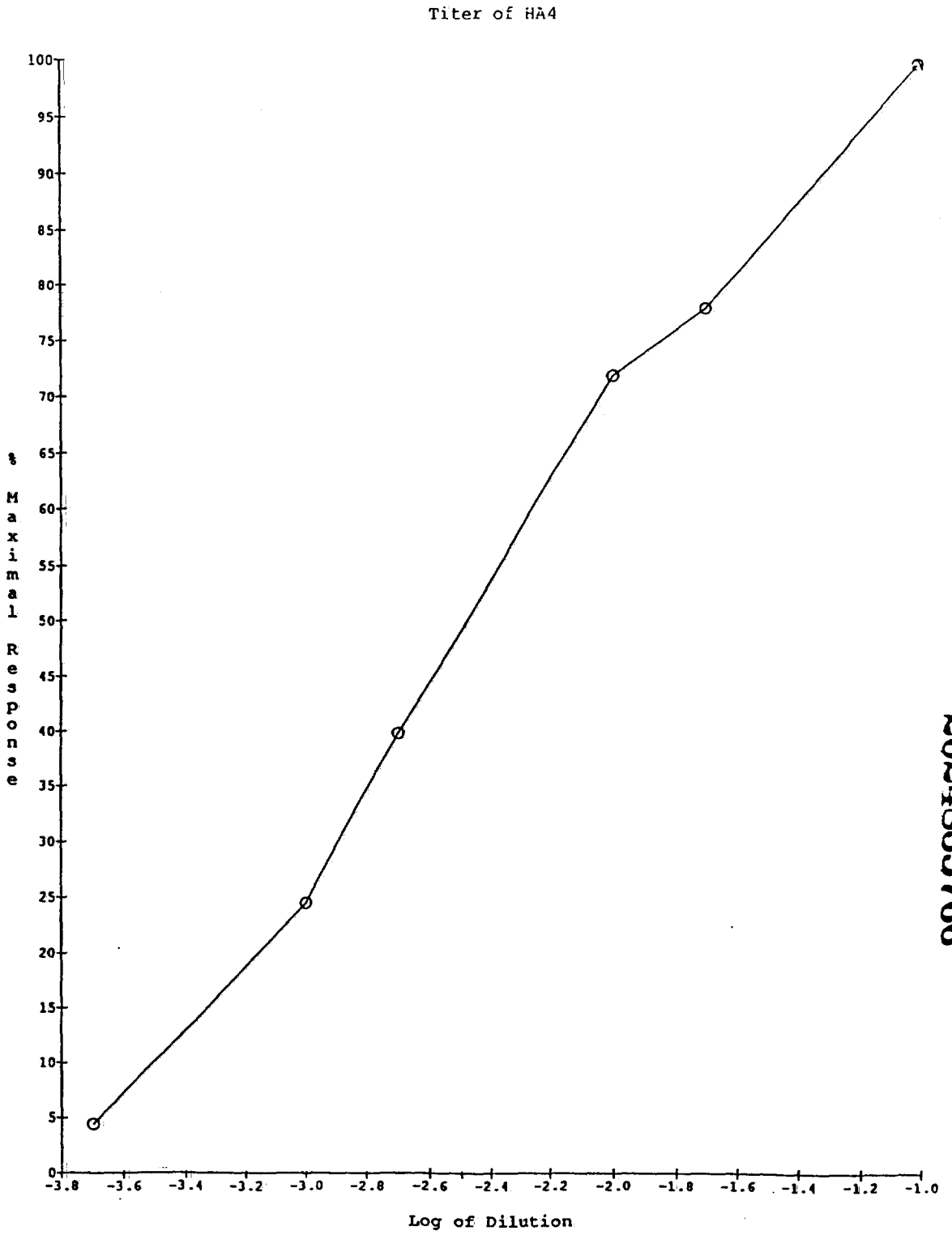

FIGURE 2

Graphical representation of the absorbance of each HA4 dilution plotted as its percent of the maximum absorbance (determined in the absense of nicotine) versus the log of the sample dilution.

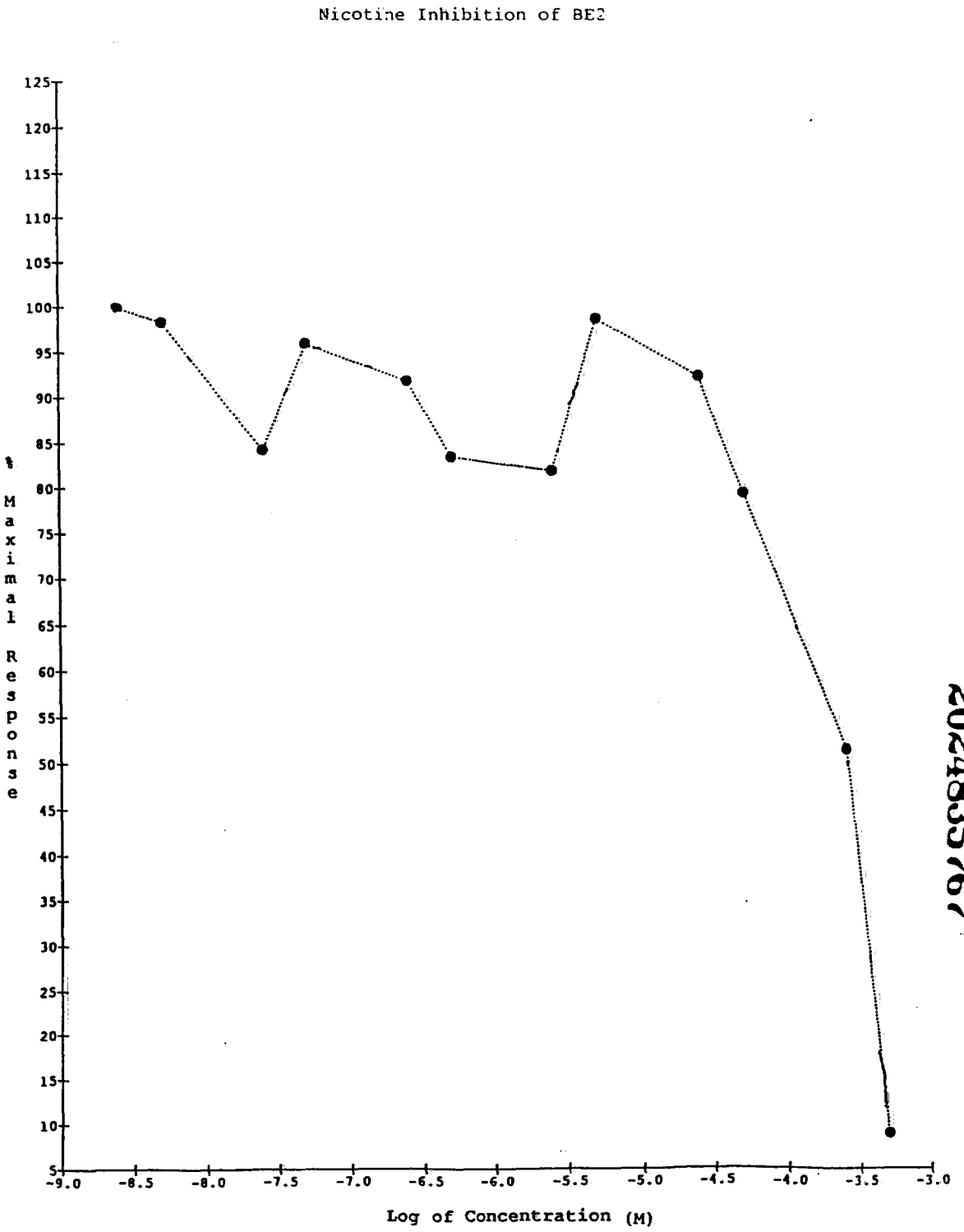

FIGURE 3

Graphical representation of the inhibition of sample BE2 with nicotine. Percent maximal response of each dilution is plotted versus the log of the nicotine concentrations used in the ELISA.

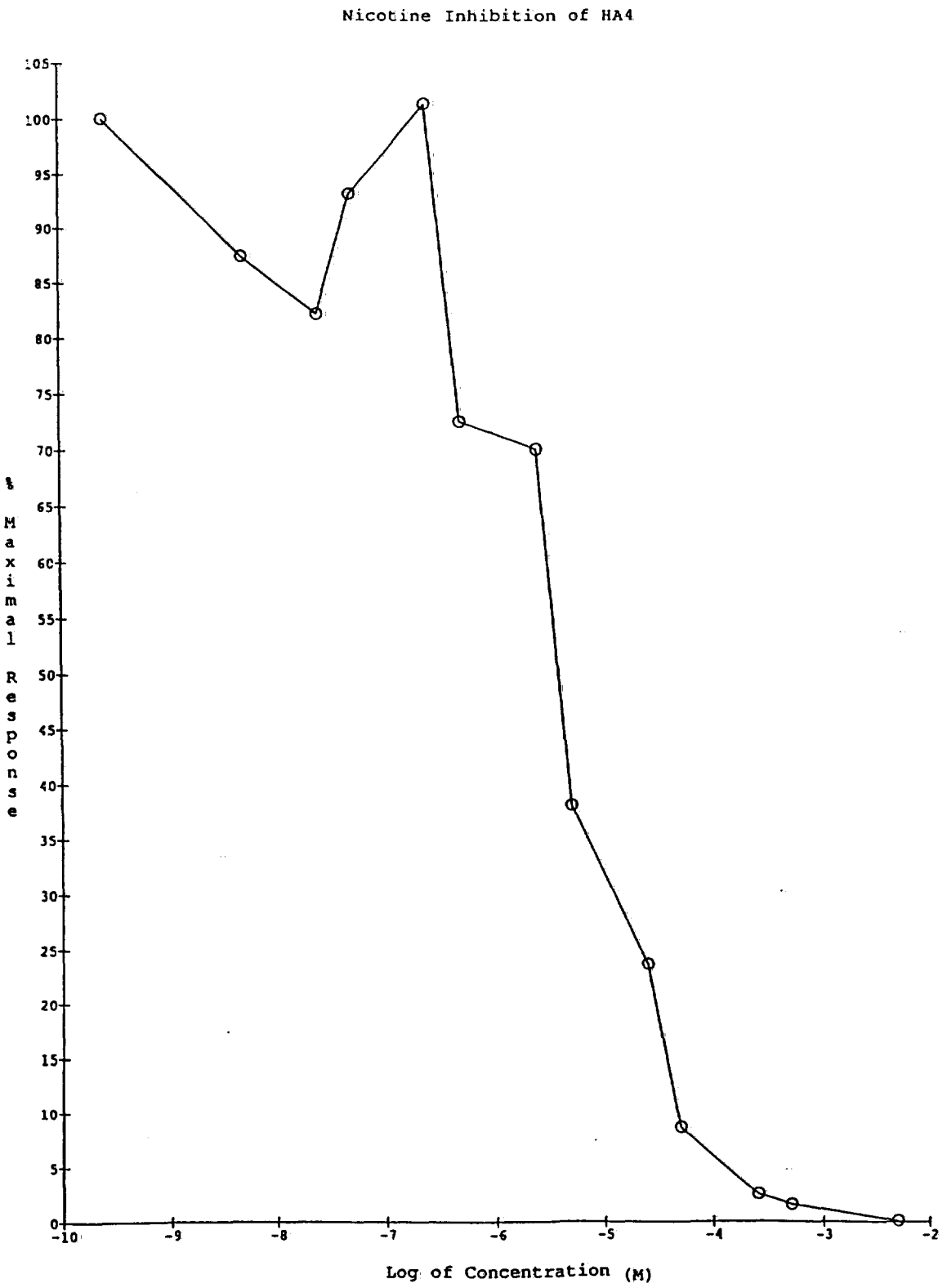

FIGURE 4

Graphical representation of the inhibition of sample HA4 with nicotine. Percent maximal response of each dilution is plotted versus the log of the nicotine concentrations used in the ELISA.

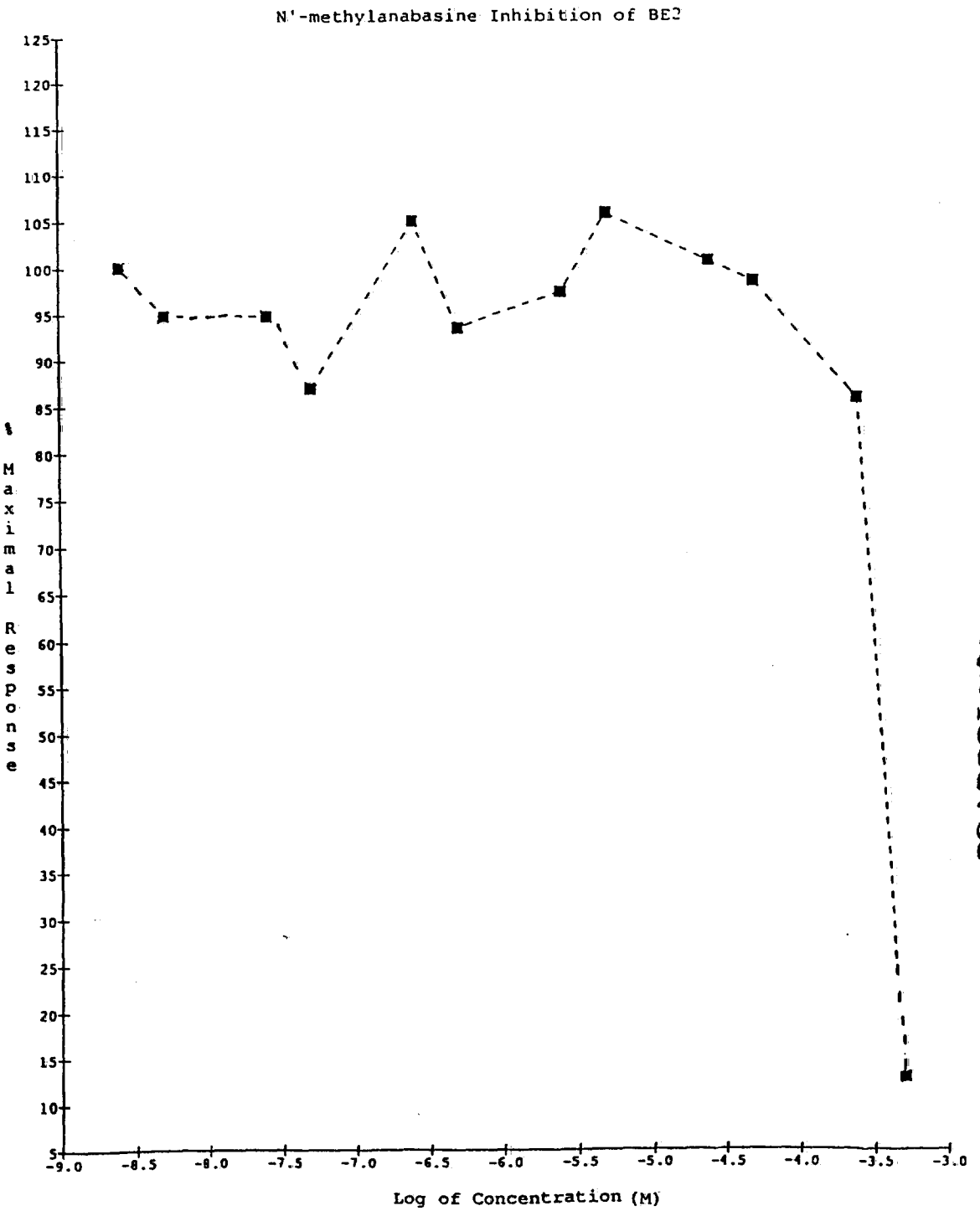

FIGURE 5

Graphical representation of the inhibition of sample BE2 with n'-methylanabasine. Percent maximal response of each dilution is plotted versus the log of the nicotine concentrations used in the ELISA.

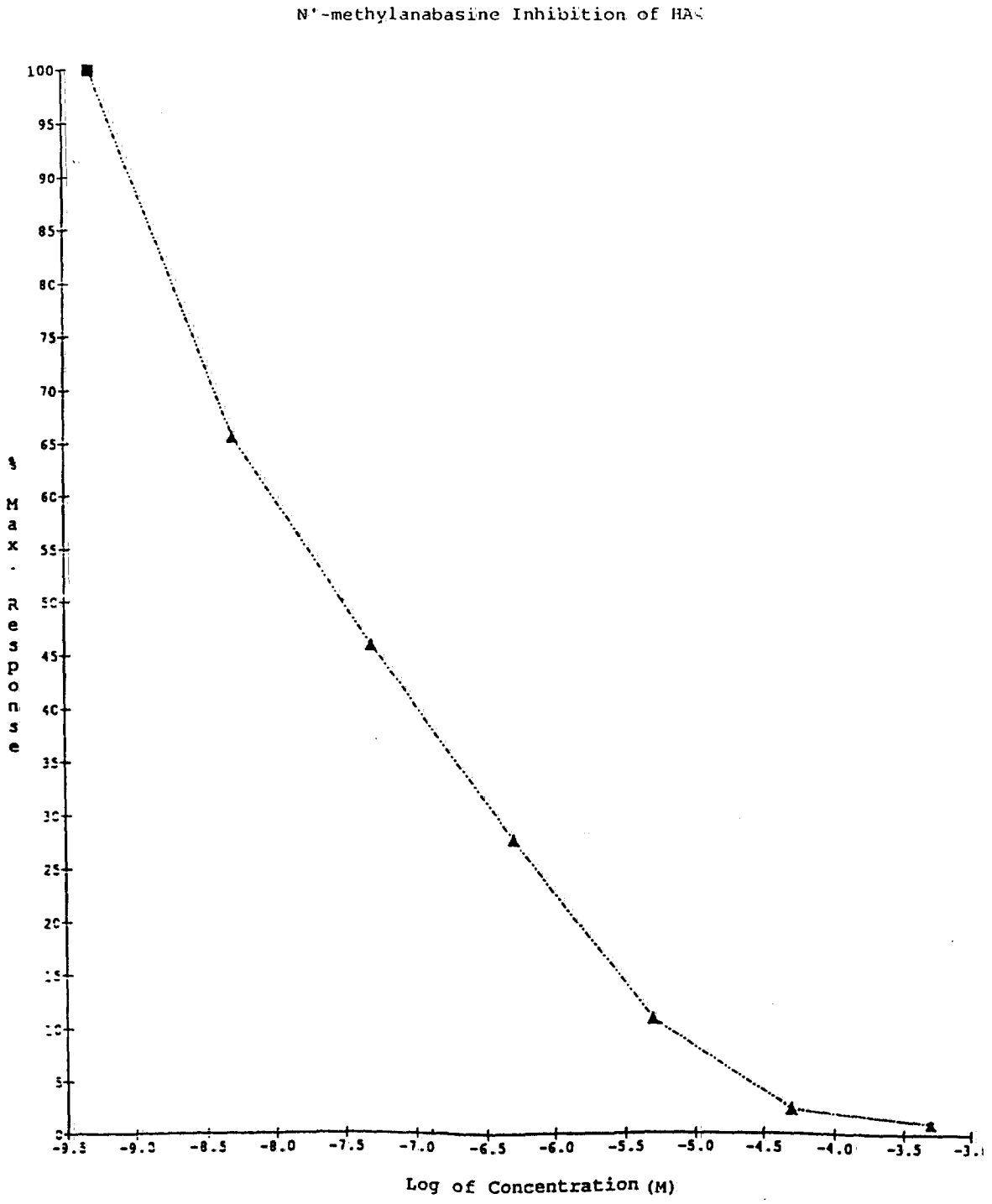

FIGURE 6

Graphical representation of the inhibition of sample HA4 with n'-methylanabasine. Percent maximal response of each dilution is shown plotted versus the log of the nicotine concentrations used in the ELISA.